PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Matilde Bustos DE ABAJO, et al
Serial No.: 10/798,219 Group No.: 1633
Filed: March 11, 2004 Examiner: Anne Marie Sabrina Wehbe
For: USE OF CARDIOTROPHIN IN LIVER DISEASES

Attorney Docket No.: U 015070-8

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.132

I, Jesús PRIETO VALTUENA, hereby declare as follows;

1. I am a co-inventor of the invention described and claimed in the above identified patent application (hereinafter "the application"). I make this declaration in support of the application. A copy of my curriculum vitae is annexed hereto as Exhibit 1.

2. I understand that an issue has arisen in the prosecution of the application as to the amount of experimentation that would have been required, as of the application filing date and based on the guidance in the application and the state of the prior art, for a person of skill in the art to devise a dosage/regimen for administration of CT-1 protein to an animal whose liver had experienced a loss of functional liver cells, and which administration would stimulate hepatic regeneration in the animal. I shall address this issue below, but first note that, as of the application filing date, a person of skill in the art to which the application pertains would have had an advanced degree in hepatology or the like and/or at least 5 years of experience working in this area. Such person would have had a knowledge of the publications discussed below.

3. As of the application filing date, there were publications available to those of skill in the art, including Jin et al (1996) Cytokine, Vol. 8 (12), 920-926 and WO95/29237, that would

have enabled one of skill in the art routinely to determine dosage amounts for administration of CT-1 protein to a subject. Both Jin and WO95/29237 (page 63-64; page 84 lines 30-34; page 87 lines 5-15) provide guidance for dosage/regimen selection. These texts offer a wide range of formulations for the preparation of therapeutic compositions of CT-1. In this connection, one of skill in the art would have understood from the specification as filed that conventional routes for CT-1 administration, such as intravenous, intraperitoneal or subcutaneous routes, may be used in the treatment and protection of the liver according to the invention. Such routes are described in WO95/29237 and/or by Jin and the skilled person would not have difficulty in using any of such routes for the treatment disclosed in the application.

4. According to WO95/29237, a typical dosage might range from 1 μg/kg/day to up to 100 mg/kg/day, preferably 10 μg/kg/day to 10 mg/kg/day (page 64 and page 84). Jin discloses a dosage of 0.5 to 2 μg/mouse twice a day, approximately 40 to 160 μg/kg/day. From these data, the skilled person could adjust a dosage for liver regeneration and protection in a subject. Starting doses in the range from 10 to 1600 μg/kg/day might be expected to be near the therapeutic doses, depending on the particular application. In fact, we have found that a dosage of 200 to 400 μg/kg/day has provided a clear protective effect in an ischemia-reperfusion protection assay conducted by inventors (see Iñiguez et al. JEM 2006; 203: 2809-2815, copy attached hereto). A similar dosage would be expected to have an effect in stimulating hepatic regeneration in a subject who has already suffered functional liver cell loss because the mechanism of operation is the same. See application at page16, line 26 to page 18, line 6.

5. On the other hand, WO95/29237 and Jin apply an administration protocol with daily administration of CT-1 during 14 or 15 days. For obtaining the therapeutic effect, both on liver hepatectomy-transplant and on acute liver damage protocols, a repeated and prolonged is not needed. This has also been confirmed by Iñiguez et al, who obtained protection with a single dose of CT-1. Therefore, possible adverse effects associated to a prolonged and repeated treatment may also be avoided.

6. I understand that the Examiner has noted that the working examples in the application use intravenous administration of Ad-CT-1 and not CT-1, and the Examiner has also noted an alleged lack of correlation (equivalence) between the use of adenoviruses and recombinant protein administration. I respectfully submit that this statement does not take into consideration the long experience those of skill in the art have had with gene transfer as an alternative to direct protein administration for a given indication and the use of gene transfer (using either viral vectors as adenoviruses or non-viral vectors), as the first option to elucidate the biological function of new proteins before the proteins are used. Gene transfer often constitutes the screening technique that helps discriminate if the biological effect of a protein (usually a molecule recently discovered for which there is no commercially available protein source) is relevant enough to enter into the laborious and costly, but routine, process of recombinant protein production. Once the functions or biological effects of the molecule have been described, one of skill in the art can predict that the use of the recombinant protein will reproduce those functions or biological effects. This is especially relevant in the case of secreted proteins (cytokines such as CT-1, for example), where the biological effect is independent of where the protein has been produced or the route of administration.

7. Indeed, we have been able to reproduce the hepatoprotective and hepatoregenerative effects obtained with Ad-CT-1 with the administration of recombinant CT-1 using routine experimental techniques. Regarding doses and routes of administrations, we followed the guidance of Jin et al and WO95/29237. Our experimental techniques in this regard are described in Exhibit 2 annexed hereto.

8. Regarding the liver tropism of adenoviruses as compared with that of the recombinant CT-1, Jin et al have shown that systemic administration of CT-1 makes the protein available to the liver and other abdominal organs such as the kidney and the spleen, so it was expected that systemic administration of recombinant CT-1 would reproduce effects observed in the liver by the use of Ad-CT-1.

9. Finally, I note that Jin et al teach that CT-1 administration for two weeks to normal mice

results in a net weight gain of several organs including the liver. This information by itself would not have constituted evidence that CT-1 has hepatoregenerative or hepatoprotective properties, but it does constitute such evidence when read in light of the disclosure in the present application.

10. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity or the application of any patent issued thereon.

December, 3 2007

DATE December 3, 2007

NAME Jesus PRIETO VALTUENA

Exhibit 1

BIOGRAPHICAL SKETCH

NAME **PRIETO, JESUS** BIRTH: Oviedo (Spain) April,6 1944 [DNI 10486228]	POSITION TITLE **PROFESSOR**

EDUCATION AND TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR	FIELD OF STUDY
University of Valladolid (Spain)	M.D.	1967	Medicine
University of Valladolid	Ph. D.	1969	Medicine (Hepatology)
University Hospital of Valladolid	Board Certifications in Gastroenterology and Internal Medicine	1969 1970	Gastroenterology/Int. Medicine
Royal Free Hospital. London (Prof. Sheila Sherlock)	Post Doctoral Studies and Clinical Assistant	1972-73	Hepatology

PROFESSIONAL EXPERIENCE

INSTITUTION AND LOCATION	TITLE	YEAR	FIELD
University Hospital of Valladolid (Spain)	Assitant Professor and Clinical Assistant	1970-72 1974-75	Int.Medicine/ Gastro/Hepatol
University Hospital of Valladolid	Associate Professor	1976	Medicine
University of Oviedo (Spain)	Professor of Medicine	1976-77	Medicine
University of Santiago de Compostela and General Hospital of Galicia (Spain)	Professor of Medicine (registration n°: A01EC1 1762)and Chairman Department of Medicine	1977-79	Int. Medicine/Gastro/Hepatol.
University of Navarra and Clinica Universitaria de Navarra (Spain)	Professor of Medicine Consultant Department of Medicine and Liver Unit	1979-85	Int. Medicine/Gastro/Hepatol
University of Navarra and Clinica Universitaria de Navarra	Professor of Medicine Director Department of Medicine and Liver Unit	1985-1996	Int. Medicine/Gastro/Hepatol
University of Navarra and Clinica Universitaria de Navarra	Professor of Medicine Director Department of Medicine and of the Division of Hepatology and Gene Therapy	1997-2006	Int. Medicine/Gastro/Hepatol
University of Navarra and Clinica Universitaria de Navarra	Professor of Medicine, Scientific Director of the Department of Medicine and Director of the Division of Hepatology and Gene Therapy	2006-	Int. Medicine/Gastro/Hepatol

AWARDS AND HONORS

- Doctor Honoris Causa . School of Medicine. University of Porto (Portugal) (2003)
- President of the Spanish Association for the Study of the Liver (2001-2005)
- Vice-President of the Spanish Association for the Study of the Liver (1985-89)
- President of the Society of Internal Medicine of Navarra, Aragon and Basque Country (1993-94)
- Member of the Scientific Committee of the European Association for the Study of the Liver (1989-92)
- Founder of the Spanish Society of Gene Therapy (2000)
- Chairman of the International Committee of the American Society of Gene Therapy (ASGT): 2006-2007
- Member of the Scientific Board of ANRS (Agencie Nationale Francaise pour la Recherche sur le SIDA et les hepatites virales - National French Agency for Investigation on AIDS and viral hepatitis) 2000-
- Expert of INSERM (Institute National Francais pour la Santé et la Recherche Medicale; National French Institute for Health and Medical Research) 2000-
- Member of the Committe of Experts of the Spanish Ministry of Health for evaluation of Interferon therapy. 1998
- Expert of the Spanish Ministry of Health for evaluation of new drugs (Agencia Española del Medicamento) 2000-
- Member or ex- member of the Editorial Committee of Gastroenterology, Hepatology Research, Journal of Hepatology, Alimentary Tract Pharmacology and Therapeutics, Revista Clinica Española, Medicina Clinica, Hepatologia y Gastroenterologia, Revista Española Enfermedades del Aparato Digestivo.
- Award "Great Prize of Bial Foundation" to Excellence in Medical Research. Lisbon 2005
- Award "Candida Medrano de Merlo" for the work on "Gene Therapy of Liver Cancer" 1996
- Award "Asturiano del Año" 2006
- Several papers were commented in different issues of *Year Book of Medicine* and deserved editorials in New England Journal of Medicine, Gastroenterology, Hepatology, Gut and other journals.
- Invited speaker in international symposia and meetings of different national Societies of Hepatology and Gastroenterology such as: Meeting

of the French Association for the Study of the Liver (Paris, 1993), European Association for the Study of the Liver (Naples, 1999), Spanish Society of Gastroenterology (Madrid 1997), Asian Symposium on Liver Diseases (Beijing 1999), Chilean Society of Gastroenterology (2000), European Gastroenterological Week (Brussels, 2000), Italian Association for the Study of the Liver (Rome, 2001), American Association for the Study of the Liver (Single Topic Conference, Airlie, Virginia, 2001), British Association for the Study of the Liver (London 2001), Polish Association for the Study of the Liver (Mikolajki, Poland, 2001), International Meeting on Therapy in Liver Diseases (Barcelona, 2001), Spanish Society of Internal Medicine (2002), Portuguese Society of Gastroenterology (2002), European Club of Liver Cell Biology (France, 2003), European Meeting on Liver Carcinogenesis (Mainz, 2003), Falk Liver Week (Friburg, 2003), Meeting on Chronic HCV infection (Bari, 2003), Argelian Society of Gastroenterology (2003), Lecturer at the University of Jilin (Changchun, China, 2004), International Meeting on Hepatocellular Carcinoma (Hong-Kong, 2004), Congress of the Netherland Society of Gastroenterology (2005), Falk Symposium (Friburg 2006), European School of Gastroenterology (Paris-Malmaison 2006), Monotematic EASL Conference on Genetics in Liver Disease (Modena, 2006), Dutch School of Gastroenterology (Leiden, 2006), Gene Therapy Unit. University of Alabama (Birmingham, USA, 2006), Research Retreat of the Department of Surgery. University of Zurich (Vulpera, Switzerland, 2007), Chairman of the International Symposium on Gene therapy clinical trials around the globe in Seattle (2007), Genome, Life and Human being (Rome, 2007), Eurocancer (Paris, 2007), University of Chiclayo (Peru, 2007),

Papers in international journals

1. Mercedes Reboredo, Maider Zabala, Itsaso Mauleon, Javier de Las Rivas, Florian Kreppel, Stephan Kochanek, Jesus Prieto, Ruben Hernandez-Alcoceba, M. Gabriela Kramer. Interleukin-12 inhibits drug-inducible systems in vivo. **GENE THERAPY**, 2007 (in press)
2. Maider Zabala[1], Juan José Lasarte[1,*], Pedro Berraondo[1], Christine Perret[2], Josu Sola[3], Maite Alfaro[1], Esther Larrea[1], Jesús Prieto[1,4] and M. Gabriela Kramer[1,*] Induction of regulatory T cells and immunosuppressive molecules counteracts the antitumor effect of interleukin-12-based gene therapy in a transgenic mouse model of hepatocellula carcinoma. **JOURNAL OF HEPATOLOGY** 2007 (in press)

3. Zabaleta A, Arribillaga L, Llopiz D, Dotor J, Lasarte JJ, Prieto J, Borras-Cuesta F, Esteban JI, Quer J, Vayreda F, Sarobe P. Induction of potent and long-lasting CD4 and CD8 T-cell responses against hepatitis C virus by immunization with viral antigens plus poly(I:C) and anti-CD40. **ANTIVIRAL RES.** 2007 Jan 22; [Epub ahead of print]
4. Larrea E, Riezu-Boj JI, Gil-Guerrero L, Casares N, Aldabe R, Sarobe P, Civeira MP, Heeney JL, Rollier C, Verstrepen B, Wakita T, Borras-Cuesta F, Lasarte JJ, Prieto J. Upregulation of indoleamine 2,3 dioxygenase in hepatitis C virus infection. **JOURNAL OF VIROLOGY.** 2007; 81:3662-6
5. Lasarte JJ, Casares N, Gorraiz M, Hervas-Stubbs S, Arribillaga L, Mansilla C, Durantez M, Llopiz D, Sarobe P, Borras-Cuesta F, Prieto J, Leclerc C. The extra domain A from fibronectin targets antigens to TLR4-expressing cells and induces cytotoxic T cell responses in vivo. **JOURNAL OF IMMUNOLOGY.** 2007;178:748-56
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10. Massip-Salcedo M, Rosello-Catafau J, Prieto J, Avila MA, Peralta C. The response of the hepatocyte to ischemia. **Liver Int.** 2007;27:6-16.
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12. Cardiotrophin-1 defends the liver against ischemia-reperfusion injury and mediates the protective effect of ischemic preconditioning. Iñiguez M, Berasain C, Martinez-Ansó E, Fortes P, Pennica D, Bustos M, Avila MA, Prieto J. **JOURNAL OF EXPERIMENTAL MEDICINE** 2006, 203: 2809-2815.
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41. Semliki Forest virus vectors engineered to express higher IL-12 levels induce efficient elimination of murine colon adenocarcinomas Rodriguez-Madoz J.R., Prieto J., y Smerdou C.. **MOLECULAR THERAPY**. 2005; 12: 153-163
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Jesús Prieto, Ignacio Melero, Puri Fortes* and Gloria González-Aseguinolaza. **MOLECULAR THERAPY**. 2005 ; 12:950-959

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1995-1997: "Gene Therapy of Hepatocellular Carcinoma using Suicide Genes" (Fundacion Echebano)

1999-2002: "Prevention and therapy of woodchuck hepatitis virus infection using immunization with defective recombinant adenoviruses and gene transfer by means of gene gun" CICYT. SAF 99-0084

1999-2001: Biological effects of Interferon alpha 5 : interaction with hepatitis C virus (Fundacion Echebano)

2000-2003: "Targeted vectors for cancer gene therapy: receptor and transcriptional targeting of retroviral, lentiviral, and adenoviral vectors". European Commission. QLK3-CT-1999-00364

2000-2002: "Gene Therapy of neoplastic and viral diseases of the liver" Fundación Ramon Areces

2002-2005 "Treatment of chronic hepatitis B in the woodchuck model". CICYT. SAF2002-03727.Investigador principal Cuantía de la subvención: 138.000 €

2003-2005 Red Nacional de Investigacion en Hepatologia y Gastroenterologia: New therapies in Hepatology. C03/C02. Subvencion 175.969 €

2005-2008 "Therapeutic role of Cardiotrophin-1 in liver surgery and transplantation. Molecular mechanisms involved in ischemia/reperfusion liver damage) " (Papel terapeutico de la

Cardiotrofina-1 (CT-1) en la cirugia de la reseccion hepatica y en el transplante hepatico: mecanismos moleculares implicados en el daño por isquemia/reperfusion). MEC. SAF2005-03513

Exhibit 2

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BRIEF DEFINITIVE REPORT

Cardiotrophin-1 defends the liver against ischemia-reperfusion injury and mediates the protective effect of ischemic preconditioning

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Ischemia-reperfusion (I/R) liver injury occurs when blood flow is restored after prolonged ischemia. A short interruption of blood flow (ischemic preconditioning [IP]) induces tolerance to subsequent prolonged ischemia through ill-defined mechanisms. Cardiotrophin (CT)-1, a cytokine of the interleukin-6 family, exerts hepatoprotective effects and activates key survival pathways like JAK/STAT3. Here we show that administration of CT-1 to rats or mice protects against I/R liver injury and that CT-1–deficient mice are exceedingly sensitive to this type of damage. IP markedly reduced transaminase levels and abrogated caspase-3 and c-Jun–NH_2–terminal kinase activation after I/R in normal mice but not in CT-1–null mice. Moreover, the protective effect afforded by IP was reduced by previous administration of neutralizing anti–CT-1 antibody. Prominent STAT3 phosphorylation in liver tissue was observed after IP plus I/R in normal mice but not in CT-1–null mice. Oxidative stress, a process involved in IP-induced hepatoprotection, was found to stimulate CT-1 release from isolated hepatocytes. Interestingly, brief ischemia followed by short reperfusion caused mild serum transaminase elevation and strong STAT3 activation in normal and IL-6–deficient mice, but failed to activate STAT3 and provoked marked hypertransaminasemia in CT-1–null animals. In conclusion, CT-1 is an essential endogenous defense of the liver against I/R and is a key mediator of the protective effect induced by IP.

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Ischemia-reperfusion (I/R) damage develops when liver blood flow is interrupted, or severely diminished, for a long period of time and then restarted. Ischemia may induce cell death by itself by causing ATP depletion, but mainly primes the cells for the more intense damage that occurs when the liver is reperfused (1). Upon reentry of oxygen, uncoupled dysfunctional mitochondria produce large amounts of oxygen-free radicals, intense oxidative stress, and mitochondrial permeability transition leading to cell death (1). On reperfusion activation of Kupffer cells also occurs, leading to abundant production of reactive oxygen species and proinflammatory cytokines, further enhancing organ damage (1). I/R injury can cause cell death by apoptosis or necrosis (1) depending on the intensity of ATP depletion. I/R liver damage is of great clinical importance because it can cause primary graft nonfunction after liver transplantation and may critically compromise the function of the remaining liver after major hepatic resections (2). The development of new therapeutic approaches to control I/R injury may benefit from better understanding of the defensive mechanisms set into motion in the liver when it is subjected to ischemic insults.

In the liver, and in various tissues, it has been shown that a short period of ischemia protects efficiently against subsequent I/R injury (3). This phenomenon, known as ischemic preconditioning (IP), indicates that a brief ischemic insult triggers a protective biological reaction in the liver which is associated with inhibition of proapoptotic pathways (3, 4). Although several mechanisms have been invoked, there is increasing evidence supporting that a sublethal oxidative stress, as occurs during a short

M.A. Avila and J. Prieto are senior authors on this paper.

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Vol. 203, No. 13, December 25, 2006 2809–2815 www.jem.org/cgi/doi/10.1084/jem.20061421

ischemic interval, plays a crucial role in the induction of IP (4). In this regard recent reports have demonstrated that the protective effect granted by IP on subsequent ischemic injury can be mimicked by treatment with H_2O_2 or an H_2O_2 analogue (5, 6). However, the downstream effectors of the protective action of reactive oxygen species are still not known.

Cardiotrophin (CT)-1 is member of the IL-6 family of cytokines that binds to a specific receptor that contains gp130 and leukemia inhibitory factor receptor (7). gp130 is common to the receptor complex of other members of IL-6 superfamily and is required for both ligand binding and signal transduction (7). CT-1 is expressed by both parenchymal and nonparenchymal liver cells and exerts potent antiapoptotic effects on hepatocytes (8). In these cells, as in cardiomyocytes and neurons, CT-1 activates cell survival signaling pathways including STAT3, extracellular-regulated kinase (Erk)1/2, and protein kinase B (Akt) (8–10). In the present work we have analyzed the possible role of CT-1 as a natural defense of the liver against I/R injury.

RESULTS AND DISCUSSION

Treatment with recombinant CT-1 reduces I/R liver injury

To determine if CT-1 was able to attenuate I/R injury, 400 μg/kg of body weight of recombinant rat CT-1 (rCT-1) was administered to Wistar rats 10 min before clamping the artery of the medium and left liver lobes. Samples were obtained at 6 h of reperfusion after 1 h of ischemia. We found that although untreated rats showed a marked rise of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and exhibited large areas of necrosis in the liver biopsy, those that were pretreated with CT-1 showed little variation of transaminases and no relevant histological changes in the liver parenchyma (Fig. 1, A and B). Subsequent determination of transaminases levels at 12 h of reperfusion showed maintained low values in rats pretreated with rCT-1 but high levels in untreated animals (unpublished data).

In another set of experiments rCT-1 at the dose of 800 μg/kg of body weight was given at the time of reperfusion after 1 h of ischemia. In these cases serum transaminases at 12 h of reperfusion were higher than in animals that received CT-1 before ischemia, but still there was significant ($P < 0.05$) protection compared with untreated rats (ALT: 5,726 ± 2,765 and 1,904 ± 478 in untreated and rCT-1–treated animals, respectively). These findings and our previous data showing that CT-1 was able to abrogate concanavalin A–induced hepatitis (8) indicate that this cytokine is able to exert hepatoprotective activity against diverse forms of liver damage.

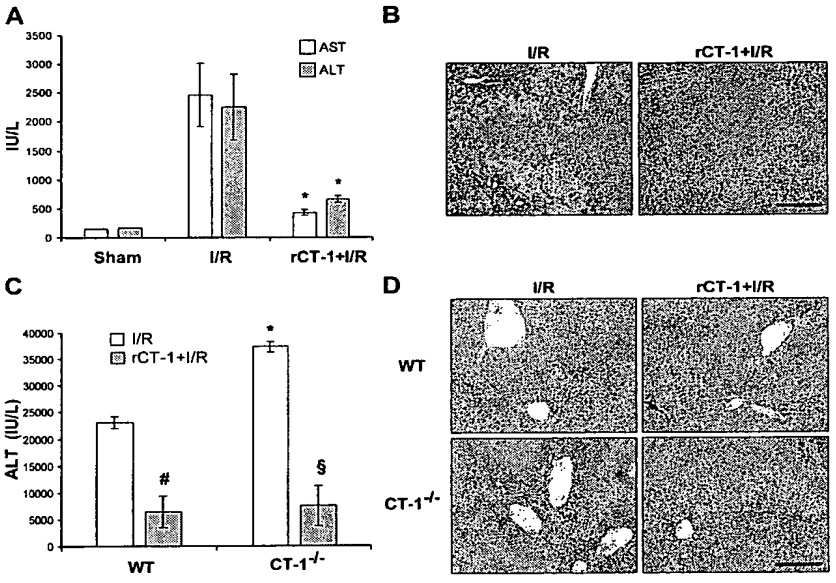

Figure 1. CT-1 defends the liver against I/R damage. (A) AST and ALT levels in the serum of rats after 1 h ischemia and 6 h reperfusion (I/R), sham-operated animals, or rats that were treated with CT-1 (400 μg/kg of weight, i.v.) 10 min before I/R. Values are means ± SD; 8 rats were used per treatment. *, $P < 0.01$ versus I/R. (B) H&E staining of representative liver tissue sections from rats that received either saline (I/R) or rCT-1 (rCT-1+I/R) before I/R as described previously. (C) ALT levels in the serum of WT and CT-$1^{-/-}$ mice after 75 min of ischemia and 3 h of reperfusion (I/R). Where indicated mice were treated with CT-1 (400 μg/kg of weight, i.v.) 10 min before I/R (rCT-1+I/R). Values are means ± SD; 5 mice were used per treatment. #, $P < 0.05$ versus untreated WT mice; §, $P < 0.01$ versus untreated CT-$1^{-/-}$ mice; *, $P < 0.05$ versus untreated WT mice. (D) H&E staining of representative liver tissue sections from WT and CT-$1^{-/-}$ mice that received either saline (I/R) or rCT-1 (rCT-1+I/R) before I/R as described previously. Bars, 100 μm.

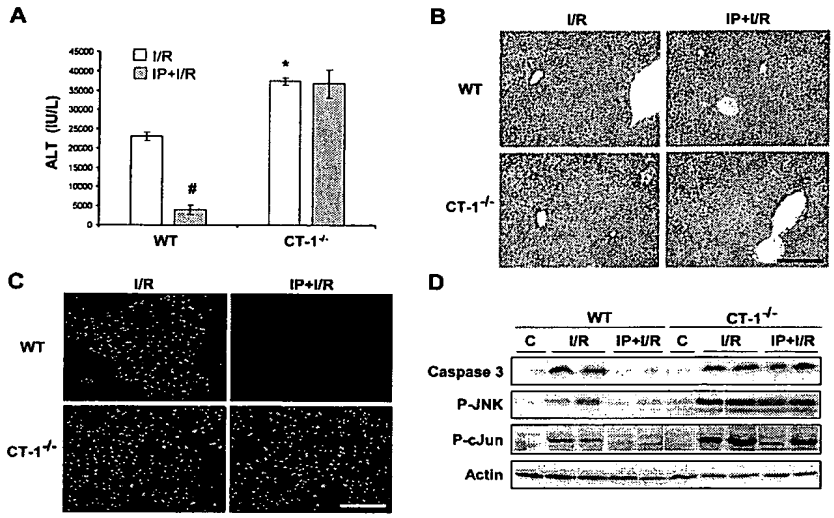

Figure 2. CT-1 is an indispensable mediator of the hepatoprotective effect induced by ischemic preconditioning. (A) ALT levels in the serum of WT and CT-$1^{-/-}$ mice after 1 h of ischemia and 3 h of reperfusion (I/R), preceded or not by ischemic preconditioning (IP) (10 min of ischemia followed by 15 min of reperfusion). Values are means ± SD; 6 mice were used per treatment. *, $P < 0.05$ versus WT mice subjected to I/R; #, $P < 0.05$ versus WT mice without IP. (B) H&E staining of representative liver tissue sections from WT and CT-$1^{-/-}$ mice after I/R preceded or not by ischemic preconditioning (IP). (C) TUNEL staining of representative liver sections from WT and CT-$1^{-/-}$ mice after I/R preceded or not by ischemic preconditioning (IP). (D) Representative Western blot analyses of active caspase 3 p-17, phosphorylated JNK, and phosphorylated c-Jun in liver samples from WT and CT-$1^{-/-}$ mice under basal conditions (C), and after I/R or I/R preceded by IP (IP+I/R). Bars, 100 μm.

CT-1 is an essential endogenous defense of the liver against I/R injury

Next, we wished to determine if CT-1 might be involved in the natural biological process that defends the liver against ischemia. To this aim we subjected CT-1–deficient mice to 75-min ischemia of the left and median lobes followed by reperfusion. We observed that the rise of serum transminases and the severity of hemorrhagic necrosis in the liver tissue that was exposed to ischemia were more intense in CT-1–null mice than in WT animals when analyzed at 3 h of reperfusion (Fig. 1, C and D). The higher sensitivity to I/R damage exhibited by CT-1–deficient mice was not caused by some abnormality different from the lack of this cytokine, because these mice were protected against I/R injury by administration of rCT-1 in the same manner as normal animals (Fig. 1, C and D). In rCT-1–treated mice (both WT and CT-1–null animals) serum ALT levels at 6 and 24 h after reperfusion remained significantly ($P < 0.05$) lower than in untreated animals (unpublished data), indicating that CT-1 treatment effectively prevented tissue injury and did not merely delay it. These data reveal an up to now unrecognized role of CT-1 as a natural defense of the liver against I/R damage.

CT-1 is a key executor of liver protection induced by ischemic preconditioning

The role played by CT-1 in liver defense against I/R prompted us to investigate if this cytokine could be a mediator of the protective biological response induced by IP. We observed that when the left and median liver lobes of normal mice were subjected to a brief period of ischemia and 15 min of reperfusion (IP) followed by 75 min of ischemia and 3 h of reperfusion (I/R injury), the histological liver lesion, the number of apoptotic hepatocyte nuclei (as estimated by the terminal deoxynucleotide transferase–mediated dUDP nick-end labeling [TUNEL] technique), and the rise of serum transaminases were markedly reduced compared with those shown by animals exposed to I/R insult without previous IP (Fig. 2, A–C). It has been reported that I/R damage is associated with phosphorylation of c-Jun–NH_2-terminal kinase (JNK), an oxidative stress–responsive kinase activated during IR liver injury (11, 12), and with activation of the proapoptotic caspase 3, a critical executor of I/R liver damage (13, 14). We found that although I/R injury caused activation of caspase 3 and phosphorylation of JNK and c-Jun in liver tissue, these events did not occur when I/R was preceded by IP (Fig. 2 D).

In contrast to WT mice, IP lacked protective effect in CT-1–deficient mice. In these animals the rise of serum transaminases, the intensity of the histological liver damage, the abundance of TUNEL-positive hepatocyte nuclei, and the activation of caspase 3, JNK, and c-Jun in hepatic tissue after I/R were similar in all mice independently of whether they had previous exposure to IP or not (Fig. 2, A–D).

STAT3 promotes antiapoptotic effects in many tissues including the liver (15), and it has been shown that the gp130-STAT3 signaling pathway mediates the hepatoprotection induced by gp130 ligands (16). There is also evidence

implicating STAT3 activation in the development of heart and brain protection associated with ischemic preconditioning (17, 18), but the mechanisms by which STAT3 is activated in response to IP remain ill understood. In the present work we found activation of STAT3 in hepatic tissue in association with liver protection against I/R injury. Thus, marked STAT3 phosphorylation together with nuclear translocation of STAT3 in hepatocytes were found in normal mice exposed to I/R challenge preceded by IP or rCT-1 administration but not in those subjected to I/R without previous treatment (Fig. 3, A and B). In contrast, IP was unable to induce STAT3 activation in CT-1–null mice in accordance with the absence of protective effect of IP in these animals. However, there was prominent phosphorylation and nuclear translocation of STAT3 in livers from CT-1–null mice after I/R when the animals were pretreated with rCT-1 (Fig. 3, A and B), a therapy that afforded protection against I/R injury.

Although it has been reported that rCT-1 may defend cardiac cells against hypoxic damage (19) and neurones against oxidative injury (20), there was no information as to whether endogenous CT-1 participates in the biological protective response elicited by IP. Our results in CT-1–deficient mice reveal that CT-1 is a critical mediator of STAT3 activation and nuclear translocation in animals exposed to IP and that CT-1 is an essential component of the hepatoprotective reaction set into motion by IP.

Administration of neutralizing antibodies to CT-1 blunt the protective effect of IP

Once we found that CT-1 was a critical component of the defensive mechanism promoted by IP, we wished to determine whether the IP could affect the expression of CT-1 in liver tissue. We observed that the hepatic levels of CT-1 protein did not change after 10 min of ischemia and 15 min of reperfusion (Fig. 4 A), suggesting that IP has no manifest effect on CT-1 synthesis. We reasoned that IP might provoke the release of preformed cytokine to the extracellular milieu to induce local autocrine and paracrine effects. To evaluate this possibility we performed an experiment consisting of the administration of neutralizing anti–CT-1 antibodies to mice subjected to IP followed by I/R. In agreement with our previous observation showing that IP was not effective in CT-1$^{-/-}$ mice (Fig. 2, A–D), neutralization of CT-1 in WT mice significantly ($P < 0.05$) blunted the protective effect of IP on I/R liver injury. This was indicated by the rise in serum AST and ALT levels, and by the activation of caspase 3 in the liver of mice treated with anti–CT-1 antibody compared with controls (IgG) (Fig. 4, B and C). Anti–CT-1 antibody administration to sham-operated mice had no effect on serum transaminases levels (unpublished data). These observations further confirmed the hepatoprotective role of CT-1, and also suggested that CT-1 must be released from intracellular stores to the extracellular milieu to mediate the hepatoprotective effects of IP. We were unable to detect by ELISA circulating CT-1 either in basal condition or after IP (unpublished data). It seems possible that CT-1 may act mainly paracrinally during IP.

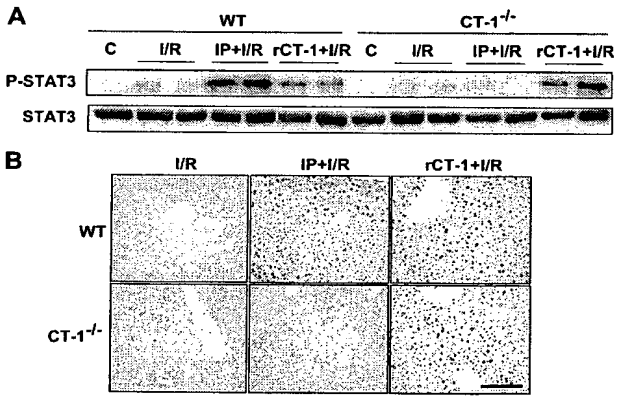

Figure 3. CT-1–deficient mice fail to activate STAT-3 in liver cells after ischemic preconditioning. (A) Representative Western blot analyses of STAT3 phosphorylation (tyr 705) and STAT3 protein levels in the liver of WT and CT-1$^{-/-}$ mice under basal conditions (C), and after I/R or I/R preceded by IP (IP+I/R) or by CT-1 treatment (rCT-1+I/R). (B) Immunohistochemical detection of phosphorylated STAT3 in representative liver sections from WT and CT-1$^{-/-}$ mice after I/R, I/R preceded by IP, or in mice treated with CT-1 (400 μg/kg of weight, i.v.) 10 min before I/R (rCT-1+I/R). Bar, 100 μm.

Oxidative stress induces the release of CT-1 from hepatocytes to extracellular milieu

It has been shown that a sublethal oxidative stress is a key event that mediates the cytoprotective effect of IP in the liver (5, 6). Sublethal concentrations of oxygen-free radicals, likely produced by Kupffer cells (6), are thought to trigger protective mechanisms on subsequent periods of ischemia; however, the identity of such mechanisms remains elusive (5). Therefore, we analyzed whether isolated hepatocytes from normal mice could release CT-1 upon exposure to a prooxidant such as the H_2O_2 analogue *tert*-butyl-hydroperoxide (tBuOOH), previously shown to mimic the effect of IP in mice (5). Western blot analysis of the supernatant of cultured hepatocytes at 30 and 60 min of incubation showed absence of CT-1 in the medium of nonstimulated cells, whereas a strong signal was observed at 60 min of incubation with tBuOOH (Fig. 4 D). A slight increase in the intracellular levels of CT-1 protein was observed after 60 min of treatment with tBuOOH (Fig. 4 D). This could be interpreted as a compensatory response to replenish intracellular stores of CT-1 after oxidative stress–stimulated release of this cytokine. From these observations it is conceivable that the oxidative stress generated during IP is responsible for the release of CT-1 to extracellular milieu. Our present data shed light on the mechanism by which oxidative stress promotes IP-induced hepatoprotection by showing that this event leads to CT-1 release, and that this cytokine is essential for the cytoprotective effect to occur because it is absent in CT-1–null mice.

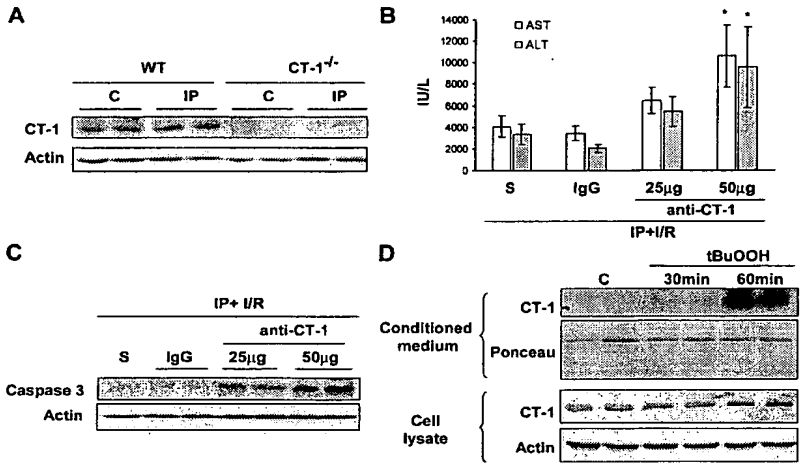

Figure 4. The protective effects of IP are blunted by anti–CT-1–neutralizing antibodies. Release of CT-1 from isolated hepatocytes upon induction of oxidative stress. (A) CT-1 is not up-regulated in IP. Representative Western blot analysis of CT-1 protein levels in the liver of WT and CT-1$^{-/-}$ mice under basal conditions (C) or after ischemic preconditioning (IP). Actin levels are shown as loading control. (B) Neutralizing antibody to CT-1 impairs the protective effect granted by IP on I/R liver injury. ALT and AST levels in the serum of mice that received saline (S), 50 μg of preimmune IgG (IgG), or increasing doses of CT-1–neutralizing antibody (anti-CT-1) 15 min before IP, and subsequently underwent I/R. *, $P < 0.05$ versus mice that received preimmune IgG. (C) Representative Western blot analyses of active caspase 3 p-17 in liver samples from mice treated as described in B. Actin levels are shown as loading control. (D) Representative Western blot analyses of CT-1 protein levels in the conditioned culture medium and cell lysates obtained from control mouse hepatocytes (C) or hepatocytes treated with tBuOOH (500 μM) for different periods of time. Ponceau S stain of Western blot membranes and actin levels are shown as loading controls.

CT-1 but not IL-6 mediates the liver defense against brief ischemia

The phenomenon of IP indicates that normal livers tolerate a short period of ischemia (and reperfusion) well by setting into motion protective mechanisms that adapt the phenotype of the tissue not only to resist this brief I/R insult but also to acquire tolerance to subsequent I/R of longer duration. The inability of CT-1–null mice to elicit protective IP suggests that CT-1 might also be essential to defend the liver against ischemia of short duration. Because IL-6 has been suggested to play a role in the modulation of I/R liver damage, and treatment with recombinant IL-6 substantially protects from ischemic liver injury (21), we decided to compare the relative role of CT-1 and IL-6 in the defense of the liver against brief I/R. To this aim we exposed the left and median lobes of the liver of WT mice, CT-1–null mice, and IL-6–null mice to 10 min of ischemia followed by 15 min of reperfusion, and at the end of this time we analyzed serum AST and ALT values and the activation of survival factors STAT3 and Akt in hepatic tissue. We observed that in both WT and IL-6–null mice AST and ALT levels showed little change with respect to control values. This resistance to short I/R exposure was associated with STAT3 and Akt activation in the two groups of animals, although with less intensity in IL-6–deficient mice (Fig. 5, A and B). In sharp contrast, in CT-1–null mice AST and ALT were considerably elevated and there was no sign of STAT3 activation and only a faint signal of phosphorylated Akt (Fig. 5, A and B). This finding reveals that CT-1, rather than IL-6, is a critical factor in the defense of the liver against ischemia of short duration.

The role of CT-1 as a natural defense against I/R liver injury and the ability of rCT-1 to protect against this form of hepatocellular damage point to potential therapeutic applications of rCT-1. New effective therapies are urgently needed for patients undergoing large hepatic resections because avoidance of I/R damage may have an important impact on postoperative morbidity and mortality by improving the function of the remaining small liver. The attractiveness of rCT-1 as a potential drug in liver surgery is enhanced by the striking increase in the number of hepatic surgical interventions during the last years owing to the frequent practice of major hepatic resections for primary or metastatic liver cancer and the increasing application of living donor liver transplantation.

MATERIAL AND METHODS

Animals. We followed University of Navarra guidelines for the use of laboratory animals. Male Wistar rats (250–275 g) were from Harlan. C57/BL6 CT-1–null mice (CT-1$^{-/-}$) (22) and WT mice were a gift from Dr. M. Selzner (Zurich University Hospital, Zurich, Switzerland). C57/BL6 IL-6–null mice (IL-6$^{-/-}$) were from The Jackson Laboratory.

Surgical procedure. Rats anesthetized with isoflurane (Abbott) were subjected to segmental hepatic ischemia followed by reperfusion (23). They were killed after 1 h of ischemia and 6 or 12 of reperfusion (I/R), and serum and liver biopsies were harvested. Sham animals were manipulated identically but without vascular clamping.

A similar I/R procedure was performed in CT-1$^{-/-}$ and WT male mice 8–10 wk old (5). The left and median lobes were occluded for 75 min, and

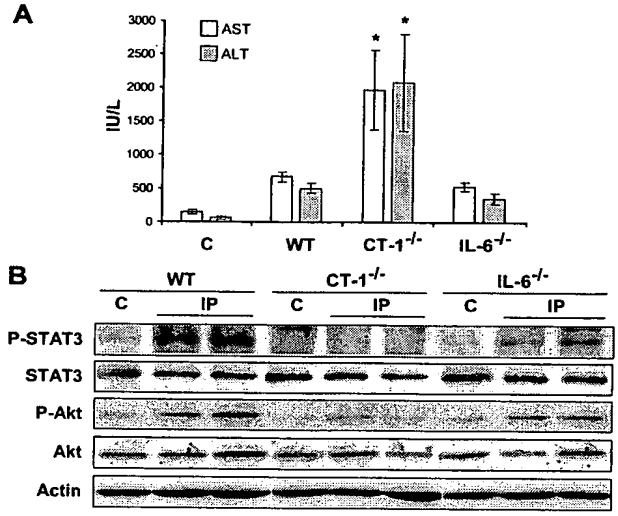

Figure 5. CT-1, and not IL-6, is the critical liver defense against a short period of ischemia. (A) AST and ALT levels in the serum of WT, CT-1$^{-/-}$, and IL-6$^{-/-}$ mice after 10 min liver ischemia followed by 15 min of reperfusion. Values are means ± SD; 5 mice were used per treatment. (B) Representative Western blot analyses of STAT3 phosphorylation (tyr 705), STAT3 protein levels, Akt phosphorylation, and Akt protein levels in the liver of WT, CT-1$^{-/-}$, and IL-6$^{-/-}$ mice under basal conditions (C) and after 10 min of liver ischemia and 15 min of reperfusion. Actin levels are shown as loading control.

after 3 h of reperfusion mice were killed for blood and tissue sampling. Where indicated IP consisting of 10 min of ischemia followed by 15 min of reperfusion was performed before I/R. Also where indicated, goat preimmune IgG (Sigma-Aldrich) or CT-1–neutralizing antibodies (R&D Systems) were administered i.v. to WT mice 15 min before IP+I/R. WT, CT-1$^{-/-}$, and IL-6$^{-/-}$ mice were subjected to brief ischemia of 10 min and killed after 15 min of reperfusion.

Liver samples were snap frozen in liquid nitrogen or formalin fixed and paraffin embedded for histological studies. Serum was used for AST and ALT aminotransferases analysis.

Rats were given 400 μg/kg body weight of rCT-1 (8) i.v. 10 min before ischemia or 800 μg/kg body weight just after declamping. In mice 400 μg/kg body weight of rCT-1 was injected 10 min before ischemia. These doses were selected based on dose–response studies performed in mice and were extrapolated to rat experiments. In these experiments, a clear protective effect was already observed at 200 μg/kg body weight of rCT-1, and protection was maximal at 400 μg/kg body weight. The rCT-1 that was used for these studies contained 0.14 pg/μg protein of LPS (*Limulus* amoebocyte lysate assay; Cambrex).

Histological analysis. Hematoxylin and eosin (H&E) staining and TUNEL assay (Roche Applied Science) were performed on paraffin-embedded liver sections as described (8, 24). Immunohistochemistry was performed on paraffin-embedded liver sections using a polyclonal anti–P-STAT3 (tyr 705) antibody (Cell Signaling) (25). The EnVision kit (Dako) was used for detection.

Western blot. Western blot was performed (24) using antibodies specific for caspase 3, P-STAT3 (tyr 705), STAT3, P-Akt, Akt (Cell Signaling), P-JNK, P–c-Jun (Santa Cruz Biotechnology, Inc), actin (Sigma-Aldrich), and CT-1 (R&D Systems).

In vitro studies. Mouse primary hepatocytes were isolated and cultured as described (25). After adhesion, hepatocytes were treated for 30 or 60 min with 500 μM of tBuOOH (Sigma-Aldrich). Cells were lysed for Western blot analyses as previously described (24). Conditioned medium was harvested and concentrated before Western blot analysis. Cell viability was not affected by tBuOOH treatment because no differences were observed in lactate dehydrogenase activity between the supernatant of control and treated hepatocytes, as assessed by the CytoTox-ONE assay from Promega.

Statistical analysis. Statistical methods used were as described previously (26). Data are means ± SD; a p value of < 0.05 was considered significant.

The technical help of Eva Petri and Sonia Gárate is acknowledged.

This work was supported by grant SAF-2005-03513 (J. Prieto) and by the agreement UTE project CIMA.

The authors have no conflicting financial interests.

Submitted: 5 July 2006
Accepted: 21 November 2006

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